

Iron bioavailability of *Lupinus rotundiflorus* seeds and roots in low-iron-diet treated rats

Biodisponibilidade do ferro das sementes e raízes do Lupinus rotundiflorus em ratos tratados com uma dieta de baixo teor de ferro

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ABSTRACT

Objective

To evaluate iron bioavailability of roots and cooked seeds of *Lupinus rotundiflorus* for human consumption using a low-iron-diet rat model.

Methods

A hemoglobin depletion–repletion test was performed using rats. A standard diet containing 8mg kg⁻¹ of iron was used. Three experimental diets were prepared based on the standard diet: 2.3g of root flour added to D1, 21.5g cooked seed flour added to D2, and 0.03g of ferrous sulfate added to D3 (control diet), adjusting iron concentration of the diets to 24mg kg⁻¹. Hemoglobin regeneration efficiency was used to measure iron bioavailability.

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Results

Hemoglobin regeneration efficiency showed values of 13.80±2.49%, 13.70±1.60% and 18.38±1.56 in D1, D2 and D3 respectively, and biological relative values of 72.8, 75.08, and 100.00% ($p<0.05$).

Conclusion

Roots and cooked seeds of *Lupinus rotundiflorus* showed potential iron bioavailability, despite being a vegetal source, which could also encourage the study of other species of lupin as a source of iron.

Keywords: Anemia. Biological availability. Hemoglobin regeneration. Iron.

RESUMO

Objetivo

Avaliar a biodisponibilidade do ferro das semente e raízes cozidas de *Lupinus rotundiflorus* para humanos usando um modelo de rato com deficiência de ferro.

Métodos

Um teste de depleção-repleção de hemoglobina foi feito usando ratos. Uma dieta base com 8mg kg⁻¹ de ferro foi usada. Partindo da dieta base, três dietas experimentais foram preparadas: D1 com 2,3g de farinha de raiz adicionada, D2 com 21,5g de farinha da semente cozida adicionada e D3 com 0,03g de sulfato ferroso adicionado (dieta controle), ajustando a concentração de ferro das dietas a 24mg kg⁻¹. A eficiência da regeneração da hemoglobina foi usada para medir a biodisponibilidade do ferro.

Resultados

Regeneração da hemoglobina apresenta valores de 13,80±2,49%, 13,70±1,60% e 18,38±1,56 na D1, D2 e D3 respectivamente e, valores biológicos relativos de 72,8, 75,08 e 100,00% ($p<0.05$).

Conclusão

As raízes e sementes cozidas do *Lupinus rotundiflorus* mostraram potencial biodisponibilidade de ferro, apesar de ser uma fonte vegetal, que poderia também incentivar o estudo de outras espécies de lupino como uma fonte de ferro.

Palavras-chave: Anemia. Disponibilidade biológica. Hemoglobinas. Ferro.

INTRODUCTION

Iron deficiency anemia is a common nutritional disorder in humans [1]. Iron deficiency is associated with low iron intake in developed countries, while in developing countries, it could be caused by low iron availability due to absorption inhibitors present in vegetable foods [2-4].

Strategies to reduce iron deficiency have been reported, such as: iron supplements, popular fortification of food products with iron, and different sources of dietary iron.

Thus, legume seeds are a good source of minerals, including iron, however they contain anti-nutritional factors (mainly oxalates and phytates), which negatively affect iron bioavailability due to

the interactions they produce during digestion and absorption [5-8].

Iron can be present in a heme form with relatively high bioavailability which can be found in red meat as part of hemoglobin and myoglobin, but it has also been reported in plants, particularly cereals, nuts, and legumes [2].

Iron in legumes is a structural part of leghemoglobin (rich in heme-iron), which is a protein homologous to hemoglobin and it is most abundant in legume nodules; however, bioavailability in these plant sources has been rarely assessed [2,8].

Lupinus are legumes that have been currently receiving considerable interest as a potential source for food ingredients. Countries

such as Australia, Poland, Germany, Chile, and Ecuador cultivate *L. albus*, *L. angustifolius*, *L. luteus* and *L. mutabilis* and consume mainly their seeds [9], incorporating them into different foods for humans or animals [10,11].

There are about 100 species of *Lupinus* in Mexico, which are widely distributed throughout the country, representing great potential due to the high protein content in the seed, 30–40%; and oil, 8–12%, depending on the species, variety, and environmental conditions [12]. However, its use is limited due to quinolizidine alkaloids that make the seed bitter and toxic. However, up to 95% of these compounds can be removed by boiling [13,14].

Due to the aforementioned, the aim of this study was to evaluate the iron bioavailability in roots and cooked seeds of *L. rotundiflorus* for human consumption using an iron-deficient rat model.

METHODS

Whole plants of *L. rotundiflorus* in fructification were harvested by hand in Jalisco, Mexico, in August 2015. Specimens were botanically identified and deposited at the *Herbario del instituto de Botánica de la Universidad de Guadalajara*, Mexico.

Mature seeds and whole roots with nodules were separated, 500g of seeds were boiled in water (ratio 1:5 w/v) at atmospheric pressure for 3 hours, with water replenishment, 500g of roots were washed with deionized water to remove soil residues, dried at 55°C for 48 hours in a forced-air stove, and ground to obtain a flour with particle size of 0.5mm in diameter for chemical analysis (n=3) and assessment of bioavailability.

The flour of the cooked seeds, roots, and diets were analyzed using the techniques of Association of Official Analytical Chemists [15]. The crude fat, crude protein, ash, and Total Dietary Fiber (TDF) content were analyzed by the enzymatic-gravimetric method. Total iron content

was determined by flame atomic absorption (GBC–AVANTA, GBC Scientific Equipment Ltd., Dandenong, Victoria, Australia) in which the ash samples were diluted in an acid solution.

For the iron bioavailability test, a AIN–93G (standard diet) with no added iron ½ IRR with iron content lower than 8 ppm was obtained from TestDiet, manufactured in Richmond, Indiana, United States [15,16].

Three experimental diets were prepared based on the standard diet: Diet one (D1) consisted of 2.3g of *L. rotundiflorus* root flour added to 100g of standar diet; Diet two (D2) of 21.5g of *L. rotundiflorus* cooked seed flour added to 100g of standard diet; while Diet three (D3) consisted of only 0.03g of ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) added to standar diet, which was considered the control diet. The amount of iron added to the standard diet content was adjusted to each of the iron sources and, once the analysis was performed, the results of the Fe concentration showed final concentrations of 24mg kg⁻¹ in diets, which is similar to the one required for rats on weaning and growth [17]. Food and deionized water was available ad libitum (Table 1).

Analysis of iron bioavailability was performed using a hemoglobin depletion-repletion assay [15] in 36 male Wistar rats aged 21 to 23 days, housed in the vivarium of the *Centro de Investigaciones en Comportamiento Alimentario y Nutrición, Centro Universitario del Sur de la Universidad de Guadalajara*. The handling of the rats was approved by the Behavior Research Center in Food and Nutrition ethics committee. The animals were weighed and placed in individual polycarbonate cages with a stainless-steel lid, in a controlled environment with a 12 hours light-dark cycle at 22°C. At the beginning of the experiment, five milliliters of blood were extracted from each rat by tail incision to determine the hemoglobin and hematocrit concentration value using the automated hematology analyzer Celly–70 Biocode Haycel.

Table 1. Composition of diets.

Ingredients (g)	Standard diet	D1	D2	D3
Cornstarch	39.75	39.75	39.75	39.75
Casein – vitamin free	20.00	20.00	20.00	20.00
Maltodextrin	13.20	13.20	13.20	13.20
Saccharose	10.00	10.00	10.00	10.00
Soy oil	7.00	7.00	7.00	7.00
Powdered cellulose	5.00	5.00	5.00	5.00
AIN-93G Min PX/no iron added	3.50	3.50	3.50	3.50
AIN-93 vitamins mix	1.00	1.00	1.00	1.00
L-cystine	0.30	0.30	0.30	0.30
Bitartrate	0.25	0.25	0.25	0.25
t-butylhydroquinone	0.001	0.001	0.001	0.001
<i>L. rotundiflorus</i> root flour		2.3	-	-
<i>L. rotundiflorus</i> cooked seed flour		-	21.5	-
FeSO ₄ ·7H ₂ O		-	-	0.03

Note: D1: Roots of *L. rotundiflorus* added to standard diet; D2: Cooked seeds of *L. rotundiflorus* added to standard diet; D3: Ferrous sulfate added to standard diet.

During the depletion period, the rats consumed the standard diet (AIN-93G), (iron lower than 8ppm) ad libitum. Each week, the hemoglobin of blood was verified to determine if the animals were anemic (<6g/100mL). Anemia was achieved after five weeks. Their weight and food consumption were individually recorded daily.

After the rats were depleted, the repletion period began. The rats were divided into three groups of 12 rats according to weight (similar weight average). Each group received a diet; D1, D2 and D3, respectively. During this period, weight and food consumption was recorded daily and hemoglobin concentration was monitored weekly until a normal level was obtained (11g/dL to 18g/dL), which took three weeks.

The percentage of iron bioavailability was calculated by the Hemoglobin Regeneration Efficiency (HRE) as the percentage of iron intake retained in hemoglobin: $HRE (\%) = [(HbFe_{final} - HbFe_{initial}) \times 100] / InFe_{To}$. Hb Fe_{final} is the iron concentration in the hemoglobin of the rats at the end of the repletion period. HbFe_{initial} is the iron concentration in the hemoglobin of the rats

at the beginning of the repletion period, and InFe To is the total iron intake of the rats during the repletion period, which was calculated as the product of the dietary iron concentration according to the amount of food each animal consumed during the experiment. HbFe was calculated considering a quantity of 0.067mL of blood/g rat body weight and hemoglobin iron content of 3.4mg Fe/g Hb. $HbFe = \text{Body weight (g)} \times \text{Hb (g/L)} \times 0.067\text{mL/g} \times 3.4\text{mg Fe/g Hb}$ [4, 18].

Biological Relative Value (BRV) expresses the use of an iron source relative to the standard reference (FeSO₄·7H₂O), which is calculated by dividing the HRE of the analyzed compound (D1 and D2) between the diet controls HRE (D3) X 100.

Statistical analysis of the three treatments of bioavailability was performed using Analysis of Variance and Tukey test. The Student's t-test was used to compare the differences between the initial and final Hb concentration means of each treatment during the repletion and the statistical software JMP® (SAS Campus Drive Cary, North Carolina, United States) was used to compare the values analyzed between the two periods.

RESULTS

Analysis of the chemical composition and iron content of the roots and cooked seeds of *L. rotundiflorus*, and, D1, D2, and D3 diets are shown in Table 2.

The protein content of the cooked seeds of *L. rotundiflorus* was higher than the whole roots (37.52±0.24 and 10.15±0.27g/100g of dry base, respectively). Similarly, the fat value was higher in cooked seeds than in the roots (6.26±0.08 and 0.3±0.03g/100g).

Nevertheless, ash and TDF content was higher in whole roots (6.3±0.14 and 70.7±0.27g/100g) than in the cooked seeds (3.04±0.1 and 27.4±0.4g/100g, respectively).

Iron concentration in cooked seeds of the species studied was 61.2±4.2mg/kg, however,

the Fe content in the root was much higher (700±6.0mg/kg).

The diets, protein, and TDF contents of g/100g of the dry matter of standard diet (18.3±0.32 and 5.0±0.1), D1 (17.0±0.12 and 6.83±0.02), and D3 (18.3±0.145.0±0.13) were similar. However, in D2 these nutrients were higher (24.0±0.56 and 10.0±0.08). Fat and ash contents (7.1±0.17 and 1.6±0.1) were similar in all standard diets: D1 (7.08±0.17 and 2.0±0.07); D2 (6.9±0.1 and 1.96±0.1); and D3 (7.1±0.1 and 1.6±0.06), respectively. D1, D2 and D3 were adjusted to 24.0mg/kg of iron.

Table 3 shows the initial and final weight, food intake, and mean weight gain during the depletion and repletion periods. The initial and final mean weights during the depletion period were 90.33±27.09 and 219.01±26.28g, with a

Table 2. Chemical composition and iron content of cooked seeds and roots of *L. rotundiflorus* and diets SD, D1, D2 y D3 (dry matter, n= 3).

Ingredients and diets	Protein g/100g	Fat g/100g	Ash g/100g	TDF g/100g	Fe mg/kg
Whole root	10.15±0.27	0.3±0.03	6.3±0.14	70.7±0.27	700±6.0
Cooked seed	37.5±0.24	6.26±0.08	3.04±0.1	27.4±0.4	61.2±4.2
Standard diet	18.3±0.32	7.1.0±0.17	1.6±0.1	5.0±0.1	8.0±0.00
D1	17.0±0.12	7.08±0.17	2.0±0.07	6.83±0.023	24.0±0.0
D2	24.0±0.56	6.9±0.1	1.96±0.1	10.0±0.08	24.0±0.0
D3	18.3±0.14	7.10±0.09	1.6±0.06	5.0±0.13	24.0±0.0

Note: Values are presented as means + standard deviation.

TDF: Total Dietary Fiber; SD: Standard Deviation; D1: Root of *L. rotundiflorus* added to standard diet; D2: Cooked seeds of *L. rotundiflorus* added to standard diet; D3: Ferrous sulfate added to standard diet.

Table 3. Initial and final weight, weight gain and total food intake of the different diets during the depletion-repletion periods in rats (N=36).

Periods	Food	Initial weight (g)	Final weight (g)	Weight gain (g)	Average food intake (g/day)
Depletion (N=36)		90.33±27.09	219.01±26.28	101.24±13.36	16.95±0.74
Repletion					
	D1	234.80±21.07*	308.00±22.51*	64.16±13.17 ^{a*}	20.23±2.04*
	D2	211.20±16.29*	264.79±32.15*	32.83±11.02 ^{b*}	20.38±2.44*
	D3	218.20±26.26*	270.00±26.98*	30.83±6.88 ^{b*}	20.23±1.82*

Note: Values presented as means + standard deviation. Different letters within the same column shows statistical significant differences ($p < 0.05$) for each parameter in the repletion period. *Asterisks in the same column for the repletion period indicate statistical significant differences ($p < 0.05$).

D1: Complete root of *L. rotundiflorus* added to standard diet, (n=12); D2: Cooked seeds of *L. rotundiflorus* added to standard diet (n=12); D3: Ferrous sulfate added to standard diet (n=12).

mean weight gain of 101.27+13.36. The mean food intake was 16.95+0.74g/day.

During the repletion period, data showed a statistically significant difference in weight gain between D1 (64.16+13.17) in comparison with D2 (32.83+11.02) and D3 (30.83+6.88).

The rats in D1 showed statistically higher difference ($p<0.05$) in iron intake (11.63+0.69mg) in comparison with D3 (9.24+0.04) and D2 (8.62+0.41). Hemoglobin concentration value at the beginning of the repletion period in the three rat groups showed a statistically significant difference ($p<0.05$) with respect to the final concentration of mean hemoglobin (6.86+0.50mg/dL and 13.39+1.25mg/dL), respectively. However, hemoglobin showed no statistically significant difference ($p>0.05$) among diets (Table 4).

Iron bioavailability, measured as hemoglobin regeneration efficiency, was statistically higher ($p<0.05$) in the control diet (D3: 18.38+1.56), followed by D1 (13.80+2.5%) and D2 (13.70+1.6%), with no statistically significant differences between these two groups ($p>0.05$).

As for the biological relative value, considering the ferrous sulfate as 100.0%, statistically significant differences were not found between diets with roots and cooked seeds (values of 75.08% and 72.8%, respectively).

DISCUSSION

The protein content decreases with cooking, as previous studies have reported in the

seeds in this specie [19]. Nevertheless, the values found in the present research were higher than the cooked seeds of *L. mexicanus* (33.1g/100g) and lower than the boiled seeds of *L. montanus* (42.2), *L. elegans* (43.3), *L. exaltatus* (43.4) and *L. campestris* (49.7) [19,14].

The fat value of the cooked seeds of *L. rotundiflorus* is in agreement with other authors [19] who have reported fat values of 5.41 to 8.2g/100g in other cooked seeds of wild lupines. However, fat values in boiled seeds of *L. campestris* (13.2g/100g) have been reported [14] to be higher than those observed in our study.

The total dietary fiber of cooked seeds in the species in our study was similar to the one found in the raw seeds of the same species (27.9) and higher than in the *L. elegans* (21.4) and *L. montanus* (26.1), but lower in the cooked seeds of *L. mexicanus* (28.4) and *L. exaltatus* (33.0) [19]. The value reported in the raw seeds of *L. albus* was 50.4g/100g [20].

Iron value decreases with heat treatment. A previous study reported that the raw seeds of this species has an iron content of 8.3mg/kg [19]. The higher value of Fe in whole roots is because this part of the plant presents nodules, which are rich in leghemoglobin, a protein rich in iron, due to greater nitrogen fixation activity that facilitates oxygen diffusion. In spite of this, there are few reports on this mineral content in legume roots.

Table 4. Iron intake, initial and final hemoglobin, Hemoglobin Regeneration Efficiency (HRE), and Biological Relative Value (BRV) on depleted rats feed with three different iron sources during the repletion period.

Diet	Fe Intake (mg)	Initial Hb (g/dL)	Final Hb (g/dL)	Initial HbFe (mg)	Final HbFe (mg)	HRE (%)	BRV (%)
<i>Repletion</i>							
D1	11.63±0.6 ^a	7.20±1.01 ^a	13.44±0.85 ^a	1.50±0.25 ^b	3.12±0.19 ^a	13.80±2.0 ^b	75.08 ^b
D2	8.62±0.41 ^c	6.56±0.32 ^a	12.65±2.14 ^a	1.86±0.22 ^a	3.04±0.49 ^a	13.70±1.6 ^b	72.80 ^b
D3	9.24±0.04 ^b	6.83±0.30 ^a	14.10±0.82 ^a	1.51±0.07 ^b	3.21±0.18 ^a	18.38±1.5 ^a	100.0 ^a
		X=6.86±0.5	X=13.39±1.25				

Note: Different letters in the same column show significant differences ($p<0.05$). Fe in Hb was calculated: mg Fe in Hb=(weight x Hb x 0.067 x 3.4). %HRE= [Fe Hb (final) – Fe Hb (initial) x 100]/Fe Intake (mg). %BRV=HER (D+R o D+S) / ERH (SF).

D1: Complete root of *L. rotundiflorus* added to standard diet; D2: Cooked seeds of *L. rotundiflorus* added to standard diet; D3: Ferrous sulfate added to standard diet.

On the other hand, the higher protein value in D3 is because more cooked seeds of *L. rotundiflorus* were used (which is richer in protein) to adjust the iron content.

The results found in a bioavailability study [21] were lower than the ones reported here because the initial weight was 152.57 ± 2.48 and final weight was 161.90 ± 26.79 , thus there was lower weight gain (9.33 ± 4.37), but food intake was higher (28.00 ± 2.62 g/day) in the depletion period. The data differs from ours due to the difference in the age of rats (as the rats were older in the that study) as well as a different initial weight and duration of depletion period, since the study period lasted three weeks, while ours lasted five weeks [21].

In the repletion period, the difference in weight gain in D1 could have been because the initial and final weights of the group were higher (Table 3). The values found in the present study were lower than those found in another study [20], which reported a mean weight gain of 64 to 80g during the three-week repletion period, using the same standard diet, probably because the rats were of a different strain and the repletion period started at a different age.

A hemoglobin concentration of 6.33 to 7.88mg/dL at the beginning of the repletion period and 14.00 to 14.60mg/dL at the end of repletion period was observed in rats submitted to two dietary treatments with a standard diet with ferrous sulfate or micronized iron [20], which presented data similar to the ones obtained in our study.

Iron bioavailability of 10.02 to 20.55% for ferrous sulfate was also reported [21], similar to the one found in present study. Similarly, iron bioavailability of $11.40 \pm 1.4\%$ was reported in cowpea seeds (*Vigna unguiculata*) submitted to thermal treatment (steaming for 10 minutes) [22], a value lower than the one obtained in our study for the cooked seeds of *L. rotundiflorus*. However, other authors [5] have reported higher values than those found in this study for cooked beans and soybeans (21.9% and 19.9%, respectively).

The biological relative value have a good bioavailability because legume roots generally do not contain substances that interfere with iron availability, such as phytates and oxalates, as it has been reported that these substances preferentially accumulate in legume seeds.

With regard to the iron bioavailability of $28 \pm 10\%$ reported in soybean root nodules and leghemoglobin bioavailability of $19 \pm 17\%$, with a BRV of 125 and 113, respectively [2], in comparison to the ferrous sulfate (considered as 100%). It has also been shown that thermal treatments in legume seeds eliminate mineral absorption inhibition factors and denaturalize protein molecular structure, increasing their bioavailability [5,8].

CONCLUSION

Iron concentration was greater in the roots than in seeds. *L. rotundiflorus* roots, as well as the cooked seeds, showed good bioavailability when compared to the bioavailability rate of non-heme iron, which ranges from 2%–20%. If we consider that the amount of iron found in the root nodules of the legume is formed by leghemoglobin, they may be partially purified and incorporated as a source of iron in foods.

CONTRIBUTORS

All authors were involved in the concept and design of this study. EH VALDÉS-MIRAMONTES was responsible for the development of the experimental phase, preparation of the experimental diets, and analysis of bioavailability. AG MARTÍNEZ MORENO participated in training on the handling, provided the rats for the experiment, collected the data, and carried out the nutritional analyses. MA RUIZ-LOPEZ and A LÓPEZ-ESPINOZA provided substantial contributions to the concept of the study; assessed the quality of study design, and revised the written manuscript. R RODRIGUEZ-MACIAS and JF ZAMORANATERA collected, selected the samples of the *Lupin* species, and performed statistical analysis. All authors critically reviewed the manuscript and approved the final version submitted for publication.

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